



TITLE:

Induction of alloantigen-specific CD4+ T regulatory Type 1 cells by alloantigen immunization and ultraviolet-B irradiation: a pilot study in murine transplantation models with skin and cardiac allografts.

AUTHOR(S):

Hori, Tomohide; Kuribayashi, Kagemasa; Saito, Kanako; Wang, Linan; Torii, Mie; Uemoto, Shinji; Kato, Takuma

CITATION:

Hori, Tomohide...[et al]. Induction of alloantigen-specific CD4+ T regulatory Type 1 cells by alloantigen immunization and ultraviolet-B irradiation: a pilot study in murine transplantation models with skin and cardiac allografts.. Annals of transplantation 2014, 19: 519-536

ISSUE DATE:

2014-10-17

URL:

<http://hdl.handle.net/2433/193258>

RIGHT:

This paper has been published under Creative Common Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) allowing to download articles and share them with others as long as they credit the authors and the publisher, but without permission to change them in any way or use them commercially.

Received: 2014.04.19
Accepted: 2014.07.08
Published: 2014.10.17

Induction of Alloantigen-specific CD4⁺ T Regulatory Type 1 Cells by Alloantigen Immunization and Ultraviolet-B Irradiation: A Pilot Study in Murine Transplantation Models With Skin and Cardiac Allografts

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCD 1,2 Tomohide Hori
A 2 Kagemasa Kuribayashi
B 2,3 Kanako Saito
B 2 Linan Wang
B 2 Mie Torii
A 1 Shinji Uemoto
A 2 Takuma Kato

1 Department of Transplant Surgery, Kyoto University Hospital, Kyoto, Japan
2 Department of Cellular and Molecular Immunology, Mie University Graduate School of Medicine, Tsu, Japan
3 Department of Hematology and Oncology, Mie University Hospital, Tsu, Japan

Corresponding Author: Tomohide Hori, e-mail: horit@kuhp.kyoto-u.ac.jp

Source of support: This study was supported by a Grant-in-Aid for Scientific Research (C, No. 20591523) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and the Japanese Society for the Promotion of Science

Background: The use of ultraviolet (UV)-B irradiation after alloantigen immunization is unknown because previous studies focused on UV-B irradiation before immunization. Here, we investigated immunosuppressive effects induced by UV-B irradiation after immunization, and examined the phenotype of induced regulatory T cells and the possible mechanism of induction.

Material/Methods: B6 mice (H-2^b) were intravenously immunized by splenocytes from CBF1 mice (H-2^{b/d}). One week after alloantigen immunization, B6 mice received high-dose UV-B irradiation (40 kJ/m²). Four weeks after UV-B irradiation, proliferation assays (*n*=4, in each), transplantations with skin or cardiac allografts (*n*=5, in each), cytokines in mixed lymphocyte culture (*n*=6, in each), and adoptive transfer of CD4⁺ T cells to naïve B6 mice (*n*=5, in each) were performed. Mice were divided into 4 groups: untreated control, immunized control, UV-irradiated control, and an immunized and UV-irradiated group. B6C3F1 mice (H-2^{b/k}) were used as irrelevant alloantigen with immunization controls. Anti-IL-10 monoclonal antibody was used to block IL-10 before and after UV-B irradiation.

Results: Immune responses against the immunizing antigen were markedly suppressed in immunized and UV-irradiated mice in an alloantigen-specific manner. Surprisingly, CD4⁺ T cells from immunized and UV-irradiated mice produced significantly larger amounts of IL-10, in an alloantigen-specific manner. Moreover, alloantigen-specific immunosuppression via CD4⁺ regulatory T cells was transferable to naïve B6 mice. IL-10 blocking clearly abrogated alloantigen-specific immunosuppression, indicating that UV-B irradiation evoked T regulatory type 1 cells.

Conclusions: This study demonstrates for the first time that immunization and UV irradiation induces alloantigen-specific CD4⁺ T regulatory type 1 cells, and that IL-10 plays an important role for this induction.

MeSH Keywords: Allografts • Liver Transplantation • T-Lymphocytes, Regulatory • Transplantation Immunology • Ultraviolet Rays

Full-text PDF: <http://www.annalsoftransplantation.com/abstract/index/idArt/890890>

6534 — 10 89



Background

Ultraviolet (UV) light, especially the mid-wave range (UV-B, 280–320 nm), is an important environmental factor that affects human health [1–5]. Although primary carcinogenesis is the most common problem, UV irradiation impairs immune responses to oncologic and infectious antigens [1,4,6]. Paradoxically, immunosuppressive effects induced by UV irradiation may have therapeutic potential for autoimmune diseases or host-versus-graft rejection [1,3,4,7–12]. Thus, UV-B irradiation is associated with clinical benefits.

Immunosuppressants have revolutionized clinical transplantation, but also cause pan-immunosuppressive effects [13]. Regulatory T cells (Tregs) are important in immunity [14–17], and alloantigen-specific immunosuppression is critical for organ transplantation [7,10,16,18–20]. To date, our group has focused on applications of UV irradiation [21–25]. To induce alloantigen-specific Tregs, high-dose UV-B irradiation accompanied by alloantigen immunization is required [5,7,10,11,26–29]. In the late 1980s, many studies reported that antigen-specific Tregs were induced by high-dose UV-B irradiation before antigen immunization [7,10,11,26], because it was thought that UV irradiation-induced alternation/modulation of antigen-presenting cell (APC) functions were required for antigen-specific Treg induction [1,7,10,26,29–32].

The focus of our research group is to assess the application of alloantigen-specific immunosuppression in murine transplantation models. The immune effect of UV-B irradiation after alloantigen immunization has not been established. Here, we investigated the use of UV-B irradiation after alloantigen immunization to induce alloantigen-specific immunosuppression, and investigated the mechanism and phenotype of induced Tregs.

Material and Methods

Animals

UV-irradiated mice are a useful model to investigate antigen-specific immunosuppression [7,10]. C57BL/6 (B6, H-2^b), (BALB/c×C57BL/6)F₁ (CBF1, H-2^{b/d}), and (C57BL/6×C3H/HeN)F₁ (B6C3F₁, H-2^{b/k}) mice were obtained from Japan SLC (Hamamatsu, Japan). These mice were maintained in a specific pathogen-free facility for laboratory animals at Mie University Graduate School of Medicine in accordance with institutional guidelines for animal welfare. To unify age and sex, 6-week-old female mice were used at the time of the first experimental procedure.

Study design and ethical approval

Study design is summarized in Figure 1. Although graft-versus-host disease is intractable in the field of bone marrow

transplantation, host-versus-graft reaction and systemic immunoresponse are important for organ transplantation. Here, we mainly focused on organ transplantation to reduce post-transplant rejection. Therefore, we set a semi-allogeneic combination (not a full-allogeneic combination) in the model with delayed-type hypersensitivity (DTH). All experimental procedures, including animal care, were approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine (No. 3106), based on the Ethics Guidelines of the Declaration of Helsinki.

Immunization with alloantigen

Spleens were removed from naïve CBF1 mice (H-2^{b/d}). Splenocytes were freshly isolated, and resuspended in phosphate-buffered saline (PBS). A total of 2×10⁷ cells/0.5 ml of single-cell splenocytes were intravenously injected into individual age- and sex-matched naïve B6 mice (H-2^b) via the lateral tail vein.

Immunization with dendritic cells

Bone marrow-derived mature dendritic cells (DC) were obtained from naïve CBF1 mice (H-2^{b/d}). Both thighbones were flushed with Hanks' solution to obtain bone marrow cells. Red blood cells and T cells were depleted by sorting system (Dyna magnetic beads, Invitrogen, Life Technologies, Carlsbad, CA, USA). Culture medium comprised complete Roswell Park Memorial Institute (RPMI) medium (RPMI 1640, Nissui Pharmaceutical Co., Taito, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS, USA) and 0.5 mM 2-mercaptoethanol (2-ME). Sterile flat-bottomed 6-well plates (Nunc™ ΔSurface, Sigma-Aldrich, St. Louis, MO, USA) were used for cell culture. A total of 6×10⁶ cells/3 ml/well were cultured at 37°C. Granulocyte-macrophage colony-stimulating factor (GM-CSF) (500 U/0.5 ml) was added. Medium change and GM-CSF addition were performed on days 1–3, 5, and 7. Mature DC were obtained at day 8 by cell sorting (AutoMACS program; Miltenyi Biotec Inc., Auburn, CA, USA). Expression of MHC class II and CD11c were analyzed by flow cytometry, and the purity of DC was maintained at >90%. Age- and sex-matched naïve B6 mice (H-2^b) were immunized by intravenous injection of mature DC (2×10⁶ cells/0.5 ml/mouse).

UV-B irradiation after alloantigen immunization

One week after alloantigen immunization, immunized B6 mice received UV-B irradiation. The UV source was a bank of 3 unfiltered UV lamps (UVP Inc., Upland, CA, USA) with an emission spectrum in the UV-B range (280–320 nm). The mean UV-B irradiation was 2372 mJ/cm²/h. A 10-cm² area of the ventral skin was carefully shaved without injury to the abdominal wall. To prevent unevenness of UV-B irradiation, the feet of

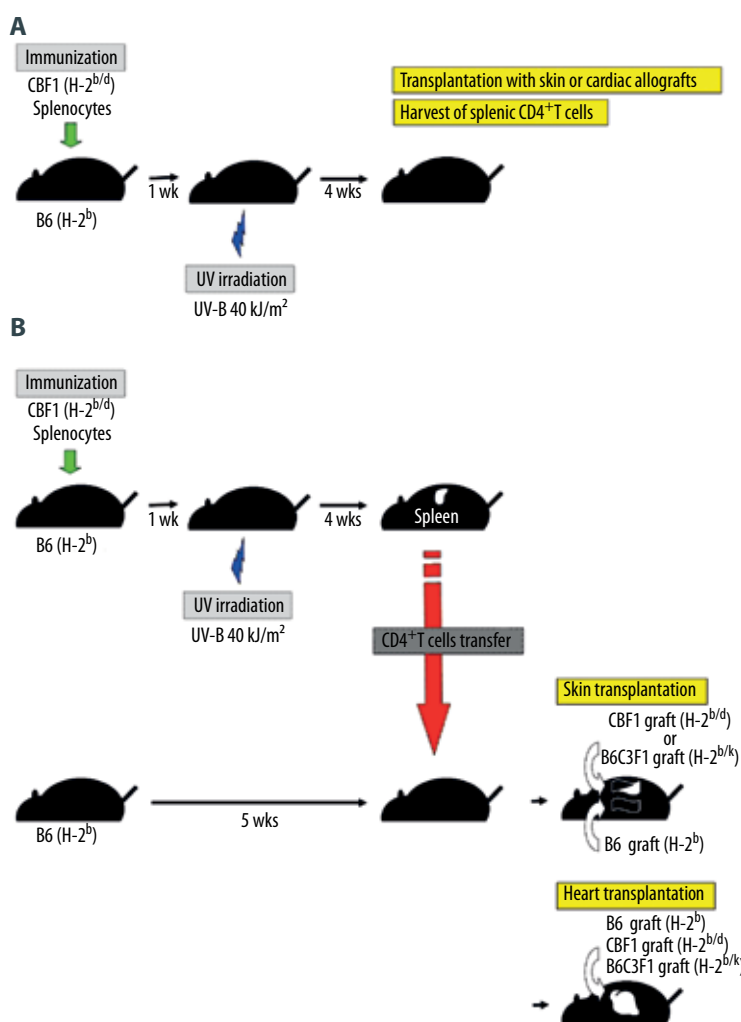


Figure 1. Study design. (A) Naïve B6 mice (H-2^b) were intravenously immunized with splenocytes from naïve CBF1 mice (H-2^{b/d}) (2×10^7 cells/0.5 ml/mouse). Immunized B6 mice received UV-B irradiation at a dose of 40 kJ/m². At 4 weeks after UV irradiation, harvest of splenic CD4⁺T cells and allograft transplantations were performed. (B) Splenic CD4⁺T cells were purified from immunized and UV-irradiated mice at 4 weeks after UV irradiation. These CD4⁺T cells were transferred into age- and sex-matched naïve B6 mice. Transferred mice received allografts immediately.

anesthetized mice were fixed to a metallic halftone plate by silk threads. Thus, the shaved abdominal wall was sufficiently extended and exposed equally to the UV lamps. The backs of mice, which were required for graft beds, were protected from UV irradiation.

Cell preparation of stimulator cells

To prevent cytokine release from stimulator T cells and proliferation of the stimulator T cells themselves, X-irradiated and T cell-depleted single splenocytes from naïve CBF1 (H-2^{b/d}) and B6C3F1 (H-2^{b/k}) mice were prepared. To deplete T cells, single splenocytes were incubated with anti-Thy1.2 magnetic beads (Anti-Mouse CD90.2 [Thy-1.2] Particles-DM; BD Biosciences, Franklin Lakes, NJ, USA), and the procedures for T cell depletion were performed using a sorting system (BD IMag system; BD Biosciences). T cell-depleted single splenocyte suspensions were X-irradiated at a dose of 28 Gy.

Mixed lymphocyte reactions

One-way allogeneic mixed lymphocyte reactions (MLR) were performed as proliferation assays in sterile flat-bottomed 96-well plates for 96 h at 37°C. All cultures were set up in triplicate. Culture medium comprised complete RPMI medium supplemented with 10% FCS, 0.5 mM 2-ME, and streptomycin. Unfractionated single splenocytes from B6 mice (5×10^5 cells) were cultured with either CBF1 or B6C3F1 stimulator cells (5×10^5 cells) in a total volume of 250 µl of culture medium in 96-well plates for 96 h at 37°C. The cultures were pulsed with 1 µCi/well of [³H]-thymidine for the last 12 h of a 96-h culture and harvested onto glass fiber filters. Proliferation was measured by [³H]-thymidine incorporation using liquid scintillation counting (counts per minute).

Skin transplantation

A whole-layer graft of tail skin was transplanted onto graft beds on the backs of recipient mice by microsurgery ($\times 16$

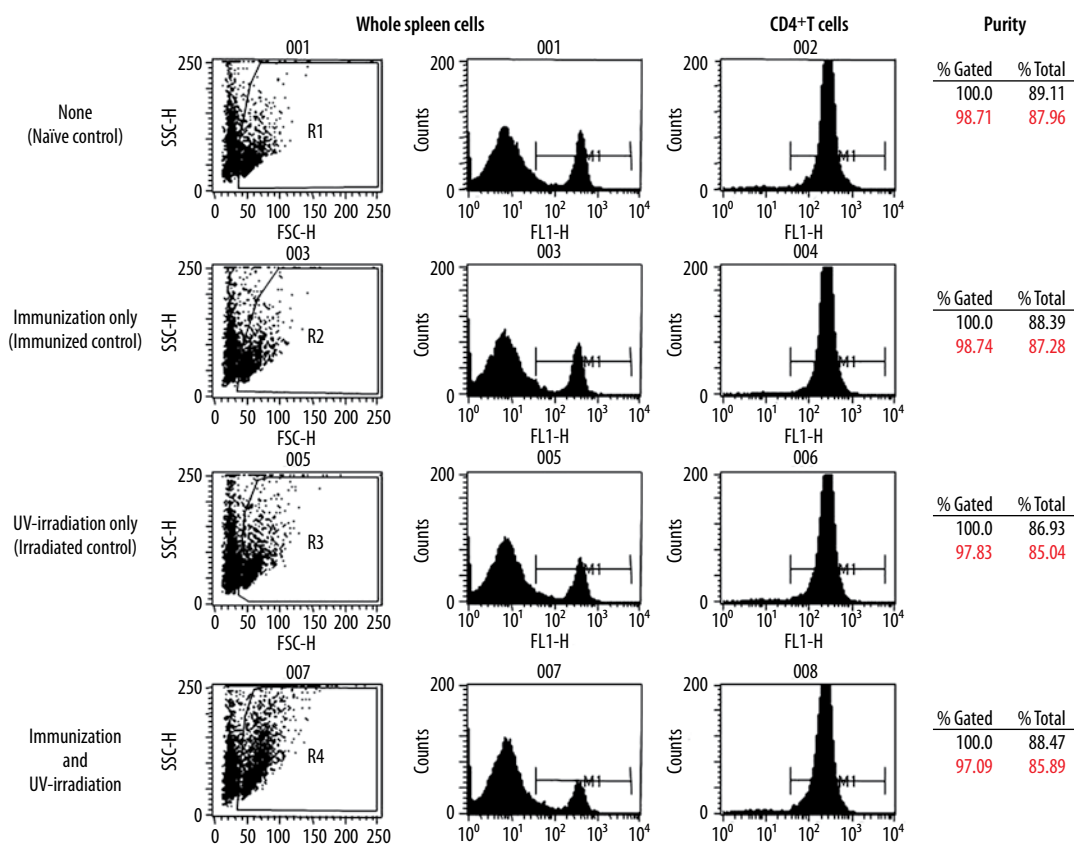


Figure 2. Purities of sorted CD4⁺T cells. Sorted splenic CD4⁺T cells routinely contained >95% of CD4⁺T cells (red).

magnification). Skin grafts were harvested from age- and sex-matched donor mice. All grafts were unified to an area of 10×5 mm to allow the quantitative uniformity of alloantigens. Under anesthesia, a syngeneic graft was transplanted onto the left- side back, while an allogeneic graft was grafted onto the right-side back. Transplanted grafts were wrapped in a sterile bandage with an antibiotic ointment. Transplanted recipient mice were placed in separate cages to avoid scratching of transplanted grafts by cage mates. They were fed with a supply of Ringer's lactate solution. Transplanted grafts were monitored every day after bandage removal at day 7. Graft rejection was defined as >90% necrosis of graft epithelium [25,33].

Heart transplantation

As the second model, to confirm immunosuppressive effects *in vivo*, heterotopic heart transplantation was employed. Cardiac grafts were harvested from age- and sex-matched donor mice. Cardiac grafts were ectopically transplanted to recipient mice. Surgical procedures, including ultra-microsurgery, were described previously [34]. Ultra-microsurgical procedures were performed under ×20 magnification (Surgical Scope M680,

Type 10445496; Leica Microsystems Inc., Bannockburn, IL, USA). Transplanted mice were placed in separate cages, and fed with a supply of Ringer's lactate solution. Graft rejection was defined as no palpable pulsation of heterotopic graft [28,35].

Cell preparation of splenic CD4⁺T cells

To prepare CD4⁺T cells, spleens were harvested from B6 mice. Single-cell suspensions of whole splenocytes (1×10⁷ cells) were incubated with CD4 microbeads (CD4 [L3T4] MicroBeads; Miltenyi Biotec, Inc.) and positively selected over separation columns (AutoMACS program; Miltenyi Biotec, Inc.). To achieve a high purity of CD4⁺T cells, splenocytes were filtrated through the separation columns twice, although the total amount of sorted CD4⁺T cells was decreased. Purities of sorted CD4⁺T cells routinely contained >95% CD4⁺T cells (Figure 2).

Mixed lymphocyte cultures (MLC)

Culture medium comprised complete RPMI medium supplemented with 10% FCS and 0.5 mM 2ME. Stimulator cells from naïve CBF1 and B6C3F1 mice were suspended in culture

medium. Responder CD4⁺T cells were purified from B6 mice. Enriched splenic CD4⁺T cell suspensions from B6 mice (1×10^6 cells/250 μ l/well) were co-cultured with stimulator cells from naïve CBF1 or B6C3F1 mice (1×10^6 cells/250 μ l/well) in sterile 96-well flat-bottomed plates at 37°C. Supernatants were harvested after 24 h for analysis of productions of interleukin (IL)-2, IL-6, and IL-8, and after 72 h for analysis of interferon (IFN)- γ , IL-4, IL-5, IL-10, IL-13, and transforming growth factor (TGF)- β production. To stimulate responder T cells, 1 μ g/mL/well of anti-CD3 monoclonal antibody (mAb) (145-2C11) was added, and supernatant was harvested after 72 h for analysis of IL-10 production. The cultures were pulsed with 1 μ Ci/well of ³H-thymidine for the last 12 h, and incorporation of ³H-thymidine was measured.

Enzyme-linked immunosorbent assay for cytokines

Concentrations of IFN- γ , IL-2, IL-4, and IL-5 in supernatants from MLC were determined by enzyme-linked immunosorbent assay (ELISA) using a pair of anti-cytokine-specific mAb, as described previously [36]. The lower detection limits for IFN- γ , IL-2, IL-4, and IL-5 were 100, 30, 10, and 10 pg/ml, respectively. Concentration of IL-6 was quantified using an ELISA kit (Mouse IL-6 ELISA; BD Biosciences). The lower limit of detection for IL-6 was 15.6 pg/ml. Concentration of IL-8 was quantified using an ELISA kit (IL-8 ELISA kit; Funakoshi Frontiers in Life Science, Tokyo, Japan). The lower limit of detection for IL-8 was 10 pg/ml. Concentrations of IL-10 and IL-13 in the supernatants of MLC were quantified using ELISA kits (Quantikine Mouse IL-10 Immunoassay and Quantikine Mouse IL-13 Immunoassay; R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's recommendations. The lower detection limits for IL-10 and IL-13 were 31.3 and 125 pg/ml, respectively. Concentrations of TGF- β were measured by ELISA kit (Mouse TGF- β 1 Platinum ELISA; eBioscience, Inc., San Diego, CA, USA). Samples were diluted 10-fold, and the lower detection limit for TGF- β was 120 pg/ml.

Serum levels of IL-10 peaked at 4 days after high-dose UV-B irradiation [27]. Peripheral blood samples were obtained at days 1-5 and 7 after UV irradiation. Serum concentrations of IL-10 were measured by ELISA.

Adoptive transfer of CD4⁺T cells

Single splenocytes were isolated from B6 mice treated with immunization and UV irradiation. Four weeks after UV irradiation, splenic CD4⁺T cells were purified and resuspended in PBS. CD4⁺T cells at concentrations of 5×10^5 , 1×10^6 , 3×10^6 , 5×10^6 , 1×10^7 , and 5×10^7 cells/100 μ l/mouse were intravenously injected into age- and sex-matched naïve B6 mice via the lateral tail vein. PBS-transferred mice served as controls.

IL-10 blocking

To block IL-10, culture medium from a hybridoma-secreting anti-IL-10 antibody (JE55-2A5, rat immunoglobulin G [IgG]) was used. The monoclonal antibody (mAb) was diluted in PBS, and a total of 250 μ g/100 μ l was intravenously injected into B6 mice, on the day before (day -1) and after (day +1) UV irradiation. In preliminary study, we confirmed the effect of this anti-IL-10 antibody in comparisons with control rat IgG (Sigma Chemical, St. Louis, MO, USA).

Statistical analysis

Results are presented as mean \pm standard deviation. The *t*-test was used for the comparison of unpaired continuous variables between groups. Survival curves were constructed by the Kaplan-Meier method, and the log-rank test was used for between-group comparisons. Statistical calculations were performed by statistical software (Stat View-J 5.0, SAS Institute Inc., Cary, NC, USA). A *p* value <0.05 was considered statistically significant, and *p* \geq 0.05 was defined as not significant (NS).

Results

Dose of UV-B irradiation

First, immunosuppressive effects were evaluated at various doses of UV-B. Mice were immunized and received various doses of UV-B at 1 week after immunization. At 1 week after UV irradiation, splenocytes were obtained from immunized and UV-irradiated mice. Proliferation assay by MLR was performed with stimulator cells presenting either the immunizing or irrelevant alloantigen. Proliferation assays by MLR were repeated 4 times (Figure 3A).

Stimulation with immunizing antigen and UV-B irradiation at 10 kJ/m² (*p*=0.0331), 20 kJ/m² (*p*<0.0001), 30 kJ/m² (*p*<0.0001), 40 kJ/m² (*p*<0.0001), 50 kJ/m² (*p*<0.0001), and 60 kJ/m² (*p*=0.0002) showed significant changes in ³H-TdR incorporation compared with control mice receiving 0 kJ/m² UV-B irradiation. Stimulation with an irrelevant antigen and UV-B irradiation at 10 kJ/m² (*p*=0.0109), 20 kJ/m² (*p*=0.0009), 30 kJ/m² (*p*=0.0008), 40 kJ/m² (*p*=0.0004), 50 kJ/m² (*p*=0.0032), and 60 kJ/m² (*p*=0.0003) showed significant changes in ³H-TdR incorporation compared with control mice receiving 0 kJ/m² UV-B irradiation.

Responses to the immunizing antigen were suppressed according to UV-B dose, although immunosuppressive effects appeared to plateau at >40 kJ/m². However, responses to the irrelevant antigen were also suppressed at 1 week after UV irradiation. In addition, a UV-B dose of 60 kJ/m² was fatal for mice. Hence, a dose of 40 kJ/m² of UV-B was used for all other experiments in this study (Figure 1)

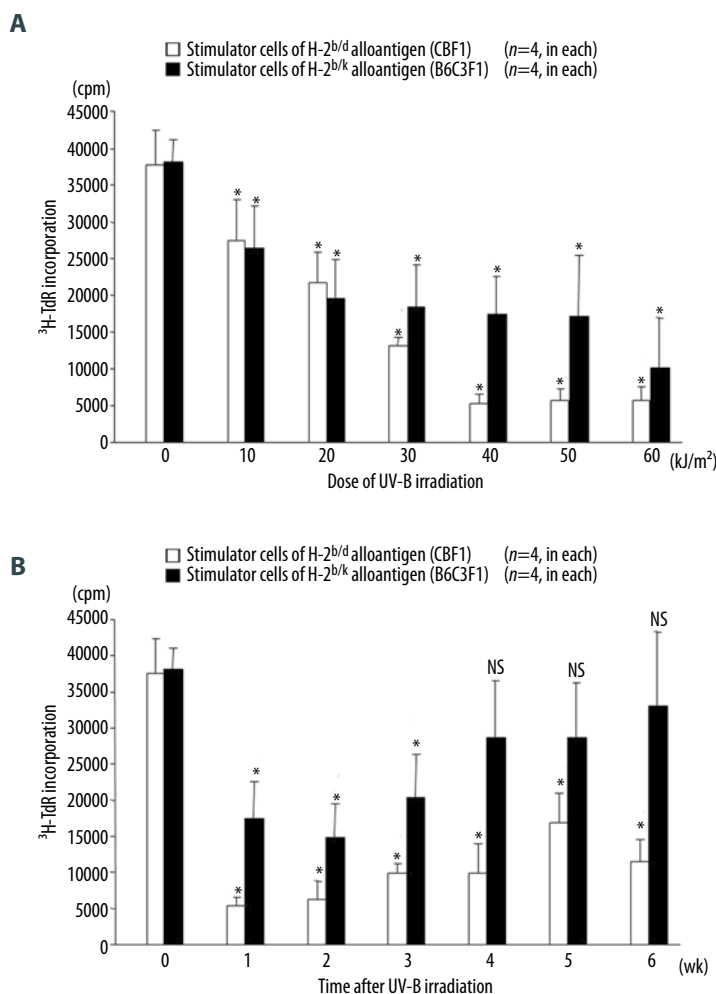


Figure 3. Proliferation assays at various doses of UV-B irradiation and at various time points after UV irradiation (A) Proliferation assays at various doses of UV-B irradiation. In each of stimulations with the immunizing or irrelevant antigen, ³H-TdR incorporations at 10-60 kJ/m² were compared with non-UV irradiated mice (0 kJ/m²), respectively. (B) Proliferation assays at various time points after UV irradiation. In each of stimulation with the immunizing or irrelevant antigen, ³H-TdR incorporations at 1-6 week(s) after UV irradiation were compared with non-UV irradiated mice (week 0), respectively. *, *p*<0.05.

Temporal immunosuppressive effects of UV-B irradiation for the irrelevant antigen

As described above, responses to an irrelevant antigen were also suppressed at 1 week after UV irradiation. The recoveries of responses to the irrelevant antigen were evaluated at various time points after UV irradiation. Immunized mice received 40 kJ/m² of UV-B at 1 week after immunization. At various time points after UV irradiation, splenocytes were obtained from immunized and UV-irradiated mice and proliferation assays by MLR were performed with stimulator cells presenting either immunizing antigen or irrelevant alloantigen (Figure 3B). Proliferation assays by MLR were repeated 4 times each.

Stimulation with the immunizing antigen induced significant differences in ³H-TdR incorporation at 1 week (*p*<0.0001), 2 weeks (*p*<0.0001), 3 weeks (*p*<0.0001), 4 weeks (*p*=0.0001), 5 weeks (*p*=0.0006), and 6 weeks (*p*<0.0001) after UV irradiation compared with non-UV irradiated mice (week 0).

Stimulation with an irrelevant antigen induced significant differences in ³H-TdR incorporation at 1 week (*p*=0.0004), 2 weeks (*p*=0.0001), and 3 weeks (*p*=0.0018) after UV irradiation compared with non-UV irradiated mice (week 0). However, ³H-TdR incorporation at 4 weeks (*p*=0.0630), 5 weeks (*p*=0.0562), or 6 weeks (*p*=0.3695) after UV irradiation was not significantly different from non-UV irradiated mice (week 0).

Responses to immunization with an irrelevant antigen disappeared at 4 weeks after UV irradiation, whereas all responses to the immunizing antigen were suppressed. Hence, a time point of 4 weeks after UV irradiation was used in this study (Figure 1)

L2 Immunosuppressive effect induced by UV irradiation alone

Transplantations with skin or cardiac allografts were performed at 1 week after UV irradiation. Mice were divided into 4 groups: (i) untreated control, (ii) immunized control (iii), UV-irradiated control, and (iv) immunized/UV-irradiated group. Skin or heart

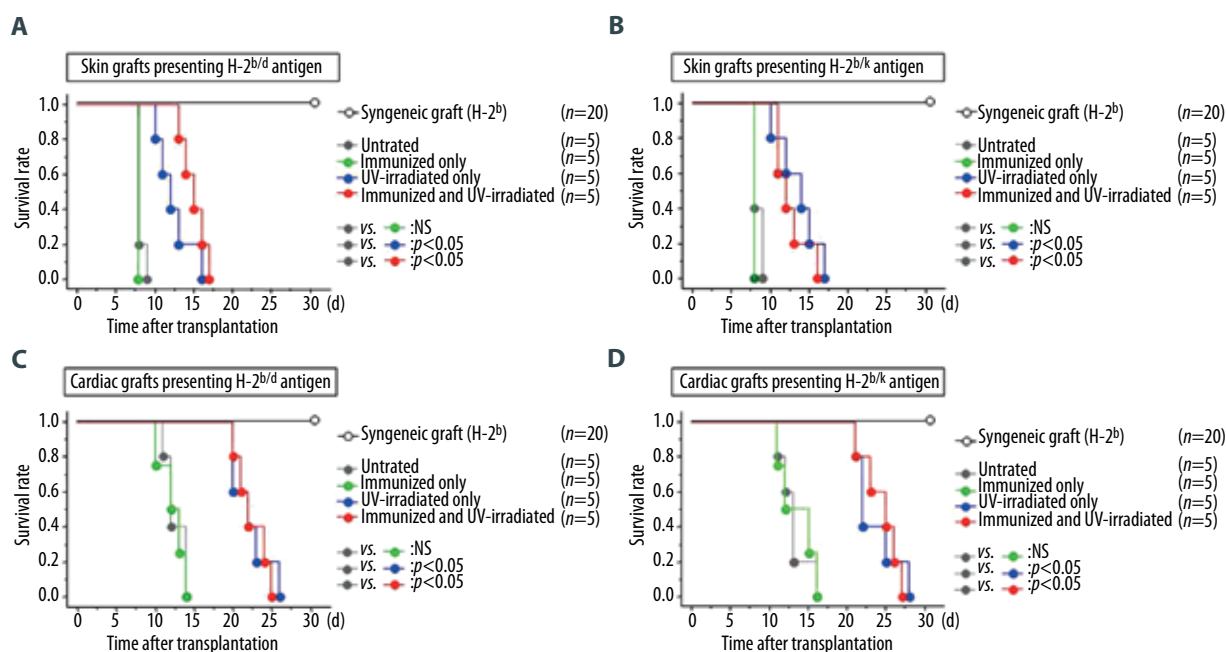


Figure 4. Allograft survival at 1 week after UV irradiation. (A) Survival of skin allografts presenting the immunizing alloantigen. (B) Survival of skin allografts presenting an irrelevant alloantigen. (C) Survival of cardiac allografts presenting the immunizing alloantigen. (D) Survival of cardiac allografts presenting an irrelevant alloantigen.

transplantations were performed in 5 mice per group. No rejections were observed following syngeneic grafts. Survival curves are shown in Figure 4.

In skin transplantation with allografts presenting the immunizing antigen, there were significant differences in allograft survival in the UV-immunized control ($p=0.0016$) and immunized/UV-irradiated group ($p=0.0016$) but not immunized controls ($p=0.3711$) compared with the untreated controls (Figure 4A). In skin transplantation with allografts presenting an irrelevant antigen, there were significant differences in allograft survival in UV-immunized control ($p=0.0023$) and immunized/UV-irradiated groups ($p=0.0023$) but not immunized controls ($p=0.1763$) compared with untreated controls (Figure 4B).

In heart transplantation with allografts presenting the immunizing antigen, there were significant differences in allograft survival in the UV-immunized control ($p=0.0062$) and immunized/UV-irradiated groups ($p=0.0062$) but not immunized controls ($p=0.7209$) compared with untreated controls (Figure 4C). In heart transplantation with allografts presenting an irrelevant antigen, there were significant differences in allograft survival in the UV-immunized control ($p=0.0051$) and immunized/UV-irradiated groups ($p=0.0051$) but not immunized controls ($p=0.8585$) compared with untreated controls (Figure 4D).

At 1 week after UV irradiation, UV-B irradiation alone showed immunosuppressive effects in transplantations with allografts regardless of which antigen was used for immunization.

Alloantigen-specific immunosuppressive effects induced by immunization and UV irradiation

Mice were divided into 4 groups: (i) untreated control, (ii) immunized control, (iii) UV-irradiated control, and (iv) immunized/UV-irradiated group. Splenic responder cells at 4 weeks after UV irradiation were cultured with stimulator cells presenting either the immunizing or irrelevant alloantigens (Figure 5). Proliferation assays by MLR were repeated 4 times each.

Immunizing antigen stimulation of the immunized/UV-irradiated group ($p=0.0006$) but not the immunized control ($p=0.2153$) or UV-irradiated control group ($p=0.2459$) showed significant differences in ³H-TdR incorporation compared with the untreated controls. However, there were no significant differences in ³H-TdR incorporation between all groups and unstimulated controls when stimulated with an irrelevant antigen.

Responses to the immunizing antigen were markedly suppressed in immunized and UV-irradiated mice, in an alloantigen-specific manner.

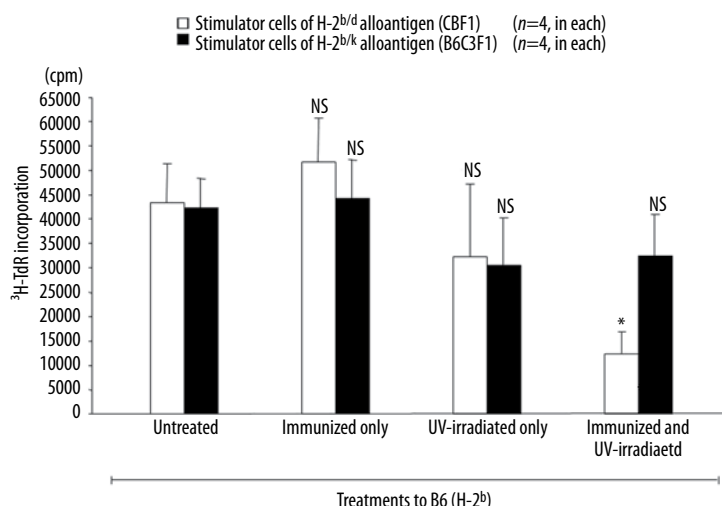


Figure 5. Proliferation assays at 4 weeks after UV irradiation. *, $p < 0.05$.

Alloantigen-specific prolongation of allograft survival induced by immunization and UV irradiation

Transplantations with skin or cardiac allografts were performed at 4 weeks after UV irradiation. Mice were divided into 4 groups, as described above. Skin or heart transplantations were respectively performed in 5 mice per group. No rejections were observed in syngeneic grafts. Survival curves are shown in Figure 6.

In skin transplantation with allografts presenting the immunizing antigen, there were significant differences in allograft survival of the immunized/UV-irradiated group ($p = 0.0052$) but not the immunized control ($p = 0.1763$) and UV-immunized control groups ($p = 0.2034$) when compared with untreated controls (Figure 6A). In skin transplantation with allografts presenting the irrelevant antigen, there was no significant difference in allograft survival of all groups when compared with the untreated controls ($p = 0.0568$) (Figure 6B).

In heart transplantation with allografts presenting the immunizing antigen, there were significant differences in allograft survival of the UV-immunized control ($p = 0.0110$) and immunized/UV-irradiated groups ($p = 0.0046$) but not of the immunized control ($p = 0.4642$) when compared with the untreated controls (Figure 6C). In heart transplantation with allografts presenting the irrelevant antigen, there were significant differences in allograft survival of the UV-immunized control ($p = 0.0120$) and immunized/UV-irradiated group ($p = 0.0262$) but not immunized controls ($p = 0.3093$) when compared with the untreated controls (Figure 6D).

At 4 weeks after UV irradiation, although immunosuppressive effects by UV-B irradiation alone were still observed in transplantation with cardiac allografts, an alloantigen-specific

prolongation of skin allograft survival was confirmed in immunized and UV-irradiated mice.

Immunization by DC

An interesting question arose from these experiments. Allografts were finally rejected, even though immunized and UV-irradiated mice showed an alloantigen-specific prolongation of allograft survival. Therefore, does immunization by mature DC cause stronger immunization and subsequent induction of greater alloantigen-specific immunosuppression in immunized and UV-irradiated mice? Using the transplant model, our preliminary study demonstrated that alloantigen immunization by intraperitoneal injection failed to induce alloantigen-specific immunosuppression or showed only weak immunosuppression (data not shown). Therefore, we used DC to provide a stronger immunization to induce greater immunosuppression.

Proliferation assays stimulated with the immunizing antigen and transplantation with skin or cardiac allografts presenting the same alloantigen were performed at 4 weeks after UV irradiation. Mice were divided into 5 groups: (i) untreated control, (ii) immunized control, (iii) immunized by DC (iv) immunized/UV-irradiated control, and (v) immunized by DC/UV-irradiated group. Proliferation assays by MLR were repeated 4 times each. Five mice were used for skin or heart transplantations. No rejections were observed in syngeneic grafts.

Proliferation assays measuring ^3H -TdR incorporation in untreated controls were examined first. There was no significant difference in ^3H -TdR incorporation between immunized controls and the immunized DC group ($p = 0.2484$) and between the immunized/UV-irradiated control and immunized by DC/UV-irradiated groups ($p = 0.7173$).

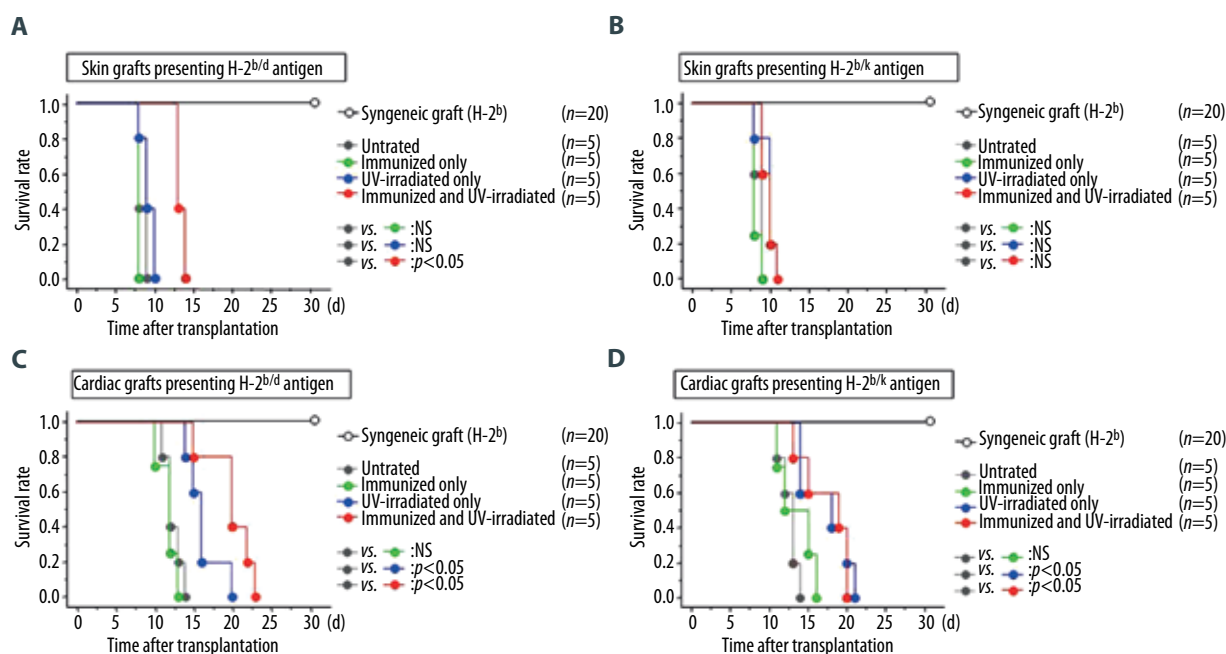


Figure 6. Allograft survivals at 4 weeks after UV irradiation. (A) Survival of skin allografts presenting the immunizing alloantigen. (B) Survival of skin allografts presenting an irrelevant alloantigen. (C) Survival of cardiac allografts presenting the immunizing alloantigen. (D) Survival of cardiac allografts presenting an irrelevant alloantigen.

Skin transplantation studies showed no significant difference in allograft survival between immunized controls and the immunized by DC group ($p=0.08741$) and between the immunized/UV-irradiated control and immunized by DC/UV-irradiated groups ($p=0.6693$). In heart transplantation studies, there was no significant difference in allograft survivals between the immunized group and immunized by DC group ($p=0.08741$) and between the immunized/UV-irradiated group and immunized by DC/UV-irradiated group ($p=0.5003$).

Although we hypothesized mature DC may have a difference in allograft survival, this was not observed experimentally.

Characterization of Treg induced by immunization after UV irradiation

To characterize whether Treg mediated alloantigen-specific immunosuppression in immunized and UV-irradiated mice, we investigated cytokine production profiles of CD4⁺T cells by alloantigen stimulation. To prepare CD4⁺T cells as responders, spleens were harvested from 4 B6 mice (H-2^b) and divided into 4 groups: (i) untreated control, (ii) immunized control, (iii) UV-irradiated control, and (iv) immunized/UV-irradiated group. CD4⁺T cells were isolated at 4 weeks after UV irradiation. Enriched CD4⁺T cells from each group were co-cultured with X-irradiated T cell-depleted splenocytes from CBF1 mice (H-2^{b/d}) or B6C3F1 mice (H-2^{b/k}). These MLCs were repeated

6 times, and supernatants were harvested for ELISA assay (Figure 7).

Proliferation in the MLC plate was measured. There was no significant difference in ³H-TdR incorporation between all groups and the untreated control when stimulated with the immunizing or irrelevant antigens (Figure 7A).

Stimulation with the immunizing alloantigen showed significant differences in IFN- γ production in the immunized/UV-irradiated group ($p=0.0010$) but not the immunized controls ($p=0.3261$) or UV-irradiated controls ($p=0.0772$) compared with untreated controls. There was no significant difference in IFN- γ production between all groups and untreated controls when stimulated with the irrelevant antigen (Figure 7B).

Stimulation with the immunizing alloantigen showed significant differences in IL-2 production in the immunized/UV-irradiated group ($p=0.0004$) but not the immunized controls ($p=0.8274$) or UV-irradiated controls ($p=0.0924$) compared with untreated controls. There was no significant difference in IL-2 production between all groups and untreated controls when stimulated with the irrelevant antigen (Figure 7C).

Concentrations of IL-4 and IL-8 were under the detection limit when cells were stimulated with the immunizing or irrelevant alloantigen (Figure 7D, 7G).

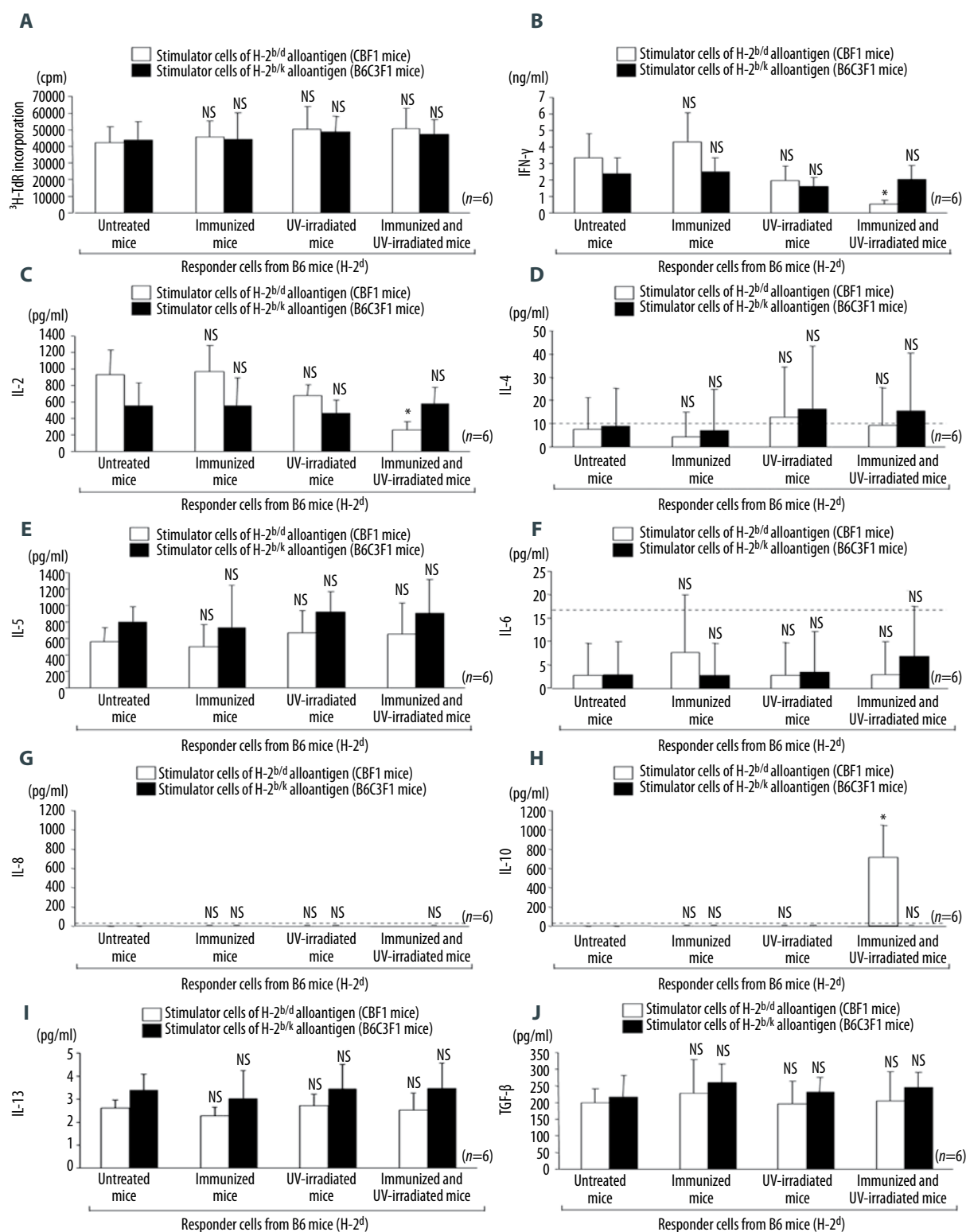


Figure 7. Cytokine profiles in MLC supernatants. (A) Proliferation of responder CD4⁺T cells. (B) IFN- γ concentration. (C) IL-2 concentration. (D) IL-4 concentration. (E) IL-5 concentration. (F) IL-6 concentration. (G) IL-8 concentration. (H) IL-10 concentration. (I) IL-13 concentration. (J) TGF- β concentration. *, $p < 0.05$; dotted line, the detection limit.

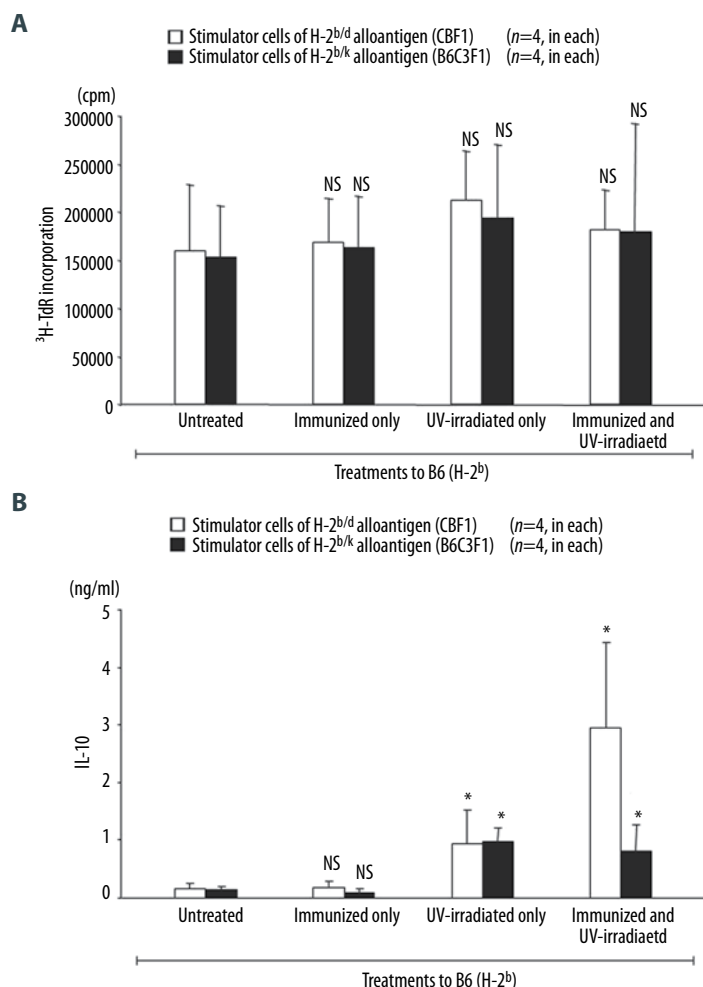


Figure 8. IL-10 production in MLC stimulated with anti-CD3 mAb. (A) Proliferation of responder CD4⁺T cells. (B) IL-10 concentration. *, $p < 0.05$.

There were no significant differences in the productions of IL-5, IL-6, and IL-13 between all groups when stimulated with the immunizing or irrelevant alloantigen (Figure 7E, 7F, 7I).

Stimulation with the immunizing alloantigen showed significant differences in IL-10 production in the immunized/UV-irradiated group ($p = 0.0004$) but not the immunized controls and UV-irradiated controls when compared with the untreated controls. There was no significant difference in IL-10 production between all groups and untreated controls when stimulated with the irrelevant antigen (Figure 7H).

There was no significant difference in TGF- β production between all groups when stimulated with the immunizing or irrelevant alloantigen (Figure 7J).

Data from the current study demonstrated that immunosuppression by immunization and UV-B irradiation did not depend on IL-4 or TGF- β , and suggested that the mechanism involved in immunosuppression was not a simple Th2 shift. CD4⁺T cells

from immunized and UV-irradiated mice produced significantly greater amounts of IL-10 but smaller amounts of IL-2 and IFN- γ compared with untreated controls when stimulated with the immunizing alloantigen but not the irrelevant alloantigen. Thus, antigen-specific Tregs induced by immunization and UV irradiation were characterized as CD4⁺ T regulatory type 1 (Tr1) cells.

Serum levels of IL-10

Serum levels of collected blood samples were under the detection limit at all time points after UV irradiation (data not shown).

Stimulation of responder T cells by anti-CD3 mAb in MLC

We stimulated cytokine production of CD4⁺T cells in MLC by anti-CD3 mAb. Mice were divided into 4 groups: (i) untreated control, (ii) immunized control, (iii) UV-irradiated control, and (iv) immunized/UV-irradiated group. MLCs with anti-CD3 mAb were repeated 4 times, and supernatants were harvested for ELISA (Figure 8).

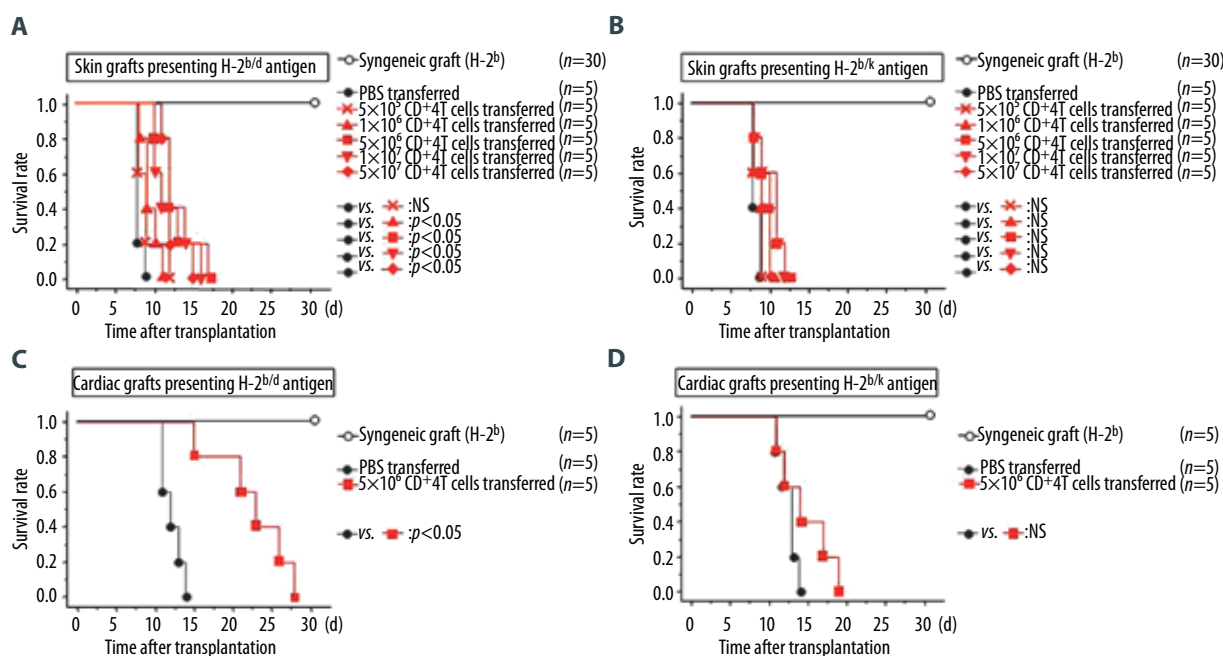


Figure 9. Allograft survivals in CD4⁺T transferred mice. (A) Survival of skin allografts presenting the immunizing alloantigen. (B) Survival of skin allografts presenting an irrelevant alloantigen. (C) Survival of cardiac allografts presenting the immunizing alloantigen. (D) Survival of cardiac allografts presenting an irrelevant alloantigen.

There was no significant difference in ³H-TdR incorporation between all groups and untreated controls when stimulated with the immunizing or irrelevant alloantigen (Figure 8A).

Stimulation with the immunizing alloantigen showed significant differences in IL-10 production in the UV-irradiated control ($p=0.0389$) and immunized/UV-irradiated groups ($p=0.0096$) but not the immunized controls ($p=0.8590$) when compared with the untreated controls. Stimulation with the irrelevant stimulator showed significant differences in IL-10 production in the UV-irradiated control ($p=0.0004$) and immunized and UV-irradiated groups ($p=0.0036$) but not the immunized controls ($p=0.3864$) when compared with the untreated controls (Figure 8B).

IL-10 production from CD4⁺T cells in immunized and UV-irradiated mice was clearly enhanced by anti-CD3 mAb stimulation.

Transferable CD4⁺ Tr1 induced by immunization and UV irradiation mediated antigen-specific prolongation of allograft survival

Based on the above results, we investigated whether alloantigen-specific immunosuppression depended on CD4⁺ Tr1 cells induced by immunization and UV irradiation. CD4⁺T cells were purified from pooled splenocytes of immunized and UV-irradiated mice at 4 weeks after UV irradiation (Figure 1). Totals

of 5×10⁵, 1×10⁶, 5×10⁶, 1×10⁷, and 5×10⁷ splenic CD4⁺T cells were transferred into naïve B6 mice. Thereafter, allografts expressing either the immunizing or irrelevant alloantigen were immediately engrafted to the transferred mice. Five transferred mice received each dose. Survival curves are shown in Figure 9.

In skin transplantations, survival of allografts presenting the immunizing alloantigen in CD4⁺T transferred mice were markedly prolonged dose-dependently ($p=0.1762$, 0.0494 , 0.0016 , 0.0016 , and 0.0016 , for CD4⁺T transfer of 5×10⁵, 1×10⁶, 5×10⁶, 1×10⁷, and 5×10⁷ cells/mouse, respectively) compared with PBS transferred mice (Figure 9A). In contrast, survival of allografts presenting the irrelevant alloantigen showed no prolongation when compared with PBS transferred mice, even in mice administered high-dose cell transfer ($p=0.5485$, 0.2055 , 0.0528 , 0.0528 , and 0.0993 , at CD4⁺T transfers of 5×10⁵, 1×10⁶, 5×10⁶, 1×10⁷, and 5×10⁷ cells/mouse, respectively) (Figure 9B).

CD4⁺T cells transferred mice from immunized/UV-irradiated mice clearly showed the dose-dependent alloantigen-specific prolongation of skin allograft survival, although the immunosuppressive effects plateaued at a dose of >5×10⁶ CD4⁺T cells/mouse.

Next, cardiac grafts were engrafted to mice receiving 5×10⁶ CD4⁺T cells. In heart transplantation, survival of allografts presenting the immunizing alloantigen in CD4⁺T transferred mice

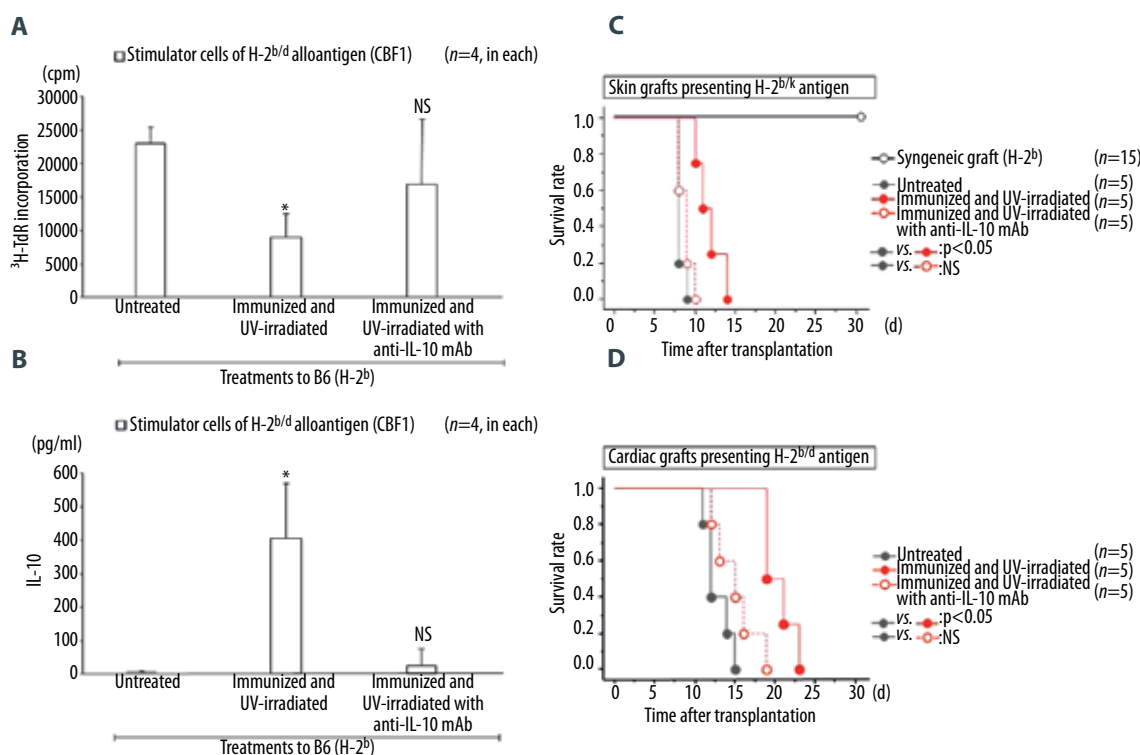


Figure 10. Abrogation of alloantigen-specific immunosuppression by blocking IL-10. (A) Proliferation assays. (B) IL-10 concentration in MLC supernatants. (C) Survival of skin allografts presenting the immunizing alloantigen. (D) Survival of cardiac allografts presenting the immunizing alloantigen. *, $p < 0.05$.

were clearly prolonged ($p = 0.0017$) compared with PBS transferred mice (Figure 9C). In contrast, survival of allografts presenting the irrelevant alloantigen showed no prolongation in CD4⁺T transferred mice when compared with PBS-transferred mice ($p = 0.2242$) (Figure 9D).

Alloantigen-specific immunosuppression induced by immunization and UV irradiation depended on CD4⁺ Tr1 cells.

Abrogation of alloantigen-specific immunosuppression by IL-10 blocking

Finally, to clarify a role of IL-10 in the induction of Tregs by immunization and UV irradiation, immunized/UV-irradiated mice were treated with anti-IL-10 mAb intravenously before (day -1) and after (day +1) UV-B irradiation. Mice were divided into 3 groups: (i) untreated control, (ii) immunized/UV irradiated control, and (iii) immunized/UV-irradiated mice with anti-IL-10 mAb treatment. MLRs and MLCs were repeated 4 times. Five mice per group received allograft transplantation, and no rejections were observed in syngeneic grafts (Figure 10).

In proliferation assay by MLRs with stimulator cells presenting the immunizing alloantigen, there was a significant difference

in ³H-TdR incorporation in the immunized/UV-irradiated controls ($p = 0.0006$) but not the anti-IL-10 mAb treated group ($p = 0.2671$) (Figure 10A).

In MLCs using responder CD4⁺T cells and stimulator cells presenting the immunizing alloantigen, there was a significant difference in IL-10 production in the immunized/UV-irradiated controls ($p = 0.0027$) but not the anti-IL-10 mAb treated group ($p = 0.3559$) (Figure 10B) when compared with the untreated controls. In transplantation with skin allografts presenting the immunizing alloantigen, there were significant differences in allograft survival of the immunized/UV-irradiated control ($p = 0.0016$) but not the anti-IL-10 mAb treated group ($p = 0.1762$) when compared with the untreated controls (Figure 10C).

In transplantation with cardiac allografts presenting the immunizing alloantigen for immunization, there were significant differences in allograft survival of the immunized/UV-irradiated control ($p = 0.0018$) but not the anti-IL-10 mAb treated group ($p = 0.1168$) when compared with the untreated controls (Figure 10D).

Treatment with anti-IL-10 mAb markedly abrogated the alloantigen-specific immunosuppression induced by immunization

and UV irradiation. These results suggests that IL-10 plays an important role in the induction of alloantigen-specific CD4⁺ Tr1 cells in immunized and UV-irradiated mice.

Discussion

UV light is one of the most important environmental factors affecting human health [1–4]. UV-B exposure induces skin tumors by a direct effect on DNA gene mutations and an indirect effect on immune responses [1,3,4]. UV-B exposure can suppress immune responses to various antigens [1,3,4]. In 1974, the first observation of the immunosuppressive effects of UV-B irradiation was reported [37]. UV-B irradiation results in the induction of highly antigenic skin cancers [2,37]. Moreover, immunosuppressive activity of UV-B-induced Tregs is highly selective, and the development of primary UV-B-induced tumors, but not other syngeneic tumors, is suppressed [2,37]. Furthermore, UV-B-induced immunosuppression could be transferred to normal syngeneic hosts by Tregs [38,39] and antigen-specific Tregs were also transferable [11,29,38,39]. Paradoxically, the capacity to modify immune responses by UV irradiation began to be used therapeutically in the 1970s [1,3].

Two models using UV-B irradiation have been developed [1,10,11,27]. Acute low-dose UV-B irradiation induces an inhibition of the local sensitization phase of contact hypersensitivity (CHS) responses to a hapten applied to UV-B irradiated skin [1,2,40]. High-dose UV-B irradiation induces inhibition of the systemic sensitization phase of CHS responses to a hapten and DTH to alloantigens applied to distant non-irradiated skin [1,2,7–11,26–28,33,41]. Both models are associated with the production of transferable antigen-specific Tregs [1,7,10,11,26,28,33]. It was suggested that the mechanisms and pathways involved in the immunosuppression of CHS and DTH induced by UV-B irradiation differ [27]. Our data clearly demonstrated that UV-B irradiation after alloantigen immunization is useful for immunosuppression in a DTH model.

High-dose UV-B irradiation induces the alternation or modulation of antigen-presenting cell (APC) functions for the induction of antigen-specific Tregs [1,3,5,7,10,26,29–31,42]. The early phase of APC functions after high-dose UV-B irradiation has been well studied [3,6,29,43,44]. UV-induced DNA damage has been recognized as the major molecular trigger for photoimmunosuppression [3,4,45]. UV exposure alters the morphology and function of epidermal Langerhans cells (LCs), which play a role in UV-B-induced immunosuppression [46]. LCs were regarded as the most important APC in the epidermis [47–49], and it was formerly believed that LCs were killed by UV irradiation. It is also believed that UV-B exposure triggers the migration of immature LCs from the skin to the draining lymph nodes, where they induce tolerance [46]. UV-B-induced migration of

mast cells to draining lymph nodes is also considered an important early step in UV-B-induced immunosuppression [43,50]. Currently, the functional role of LCs has been redefined, and UV-damaged LCs in the regional lymph nodes were required for the Treg induction [45]. Damaged but still-alive LCs will present antigen in a unusual manner, and then, this presentation will induce not effect T cells, but will affect Tregs [4]. UV-B irradiation alters the ability of APCs to activate helper T cells and UV-resistant APCs to induce Tregs [10,26,32]. This might explain the antigen specificity of UV-B-induced immunosuppression, and also why antigen immunization must follow UV-B irradiation and not vice versa [7,10,26].

CD4⁺ and CD8⁺ T cells play critical roles during allograft rejection in transplantation [15,33,51–53]. UV-induced antigen-specific immunosuppression is attributable to T cells with suppressive activity (formerly so-called ‘suppressor T cells’) [54,55], and currently these T cells are renamed as Treg [56–58]. A number of studies have investigated the phenotype and mechanism of UV-B-induced Tregs. UV-induced Tregs express CD4, CD25, and CTLA4 [56,59,60]. Most UV-induced Tregs belong to the CD4⁺ phenotype [58], and immunosuppressive effects induced by UV irradiation are mediated by CD4⁺T cells [28,61,62]. These Tregs also express the lymph node-homing receptor (CD62L) and migrate into the lymph nodes [60,63]. Therefore, UV-induced Tregs primarily inhibit sensitization. To detect the phenotype of Tregs induced by UV irradiation after immunization, we had reported that CD4⁺T cells did not express CD25, CTLA4, or Foxp3 [22–25] and that T cells sorted by only CD4 involved Treg populations with IL-10. Immunosuppressive effects induced by UV-B irradiation were mediated by CD4⁺T cells [7,11,26,28,33,61], and CD4⁺T cells induced by UV irradiation after immunization worked well in our DTH model.

CD3⁺, CD4⁺, and CD8⁺ Tregs mediated immunosuppressive effects by releasing IL-4 and IL-10 [11,33,64]. UV-B-induced immunosuppression might also be explained by a shift in the activation of T cells from a Th1 to a Th2 immune response [26,28,65,66]. CD4⁺ Th2 lymphocytes secrete pro-inflammatory cytokines (IL-4, IL-5, and IL-13) [67,68]. IL-4 is thought to promote the induction of transplantation tolerance and alloantigen-specific Tregs [17]. IL-4 also promotes both regulatory and effector T cells early in the immune response, but once alloimmune tolerance is established, IL-4 promotes the activation of effector cells to mediate rejection and does not support alloantigen-specific Tregs that could transfer specific tolerance [17]. Although IL-10 is a well-known immunosuppressive cytokine [33,69] TGF- β is a growth and differentiation factor that displays multiple functions [70]. It was suggested that the combined use of IL-10 with other inhibitory cytokines such as TGF- β might be have more effect on CD4⁺ Tregs [33,70,71]. Our results support that immunosuppression induced by UV irradiation after immunization is dependent on

CD4⁺ Tregs. However, our results regarding cytokine profiles suggest that alloantigen-specific immunosuppression induced by UV irradiation after immunization does not depend on IL-4, IL-5, IL-13, or TGF- β . As in previous reports, Tregs induced by UV irradiation before immunization showed the differences in IL-4, IL-5, and TGF- β [26,28,52,53], and our preliminary data may also explain immunosuppressive effects induced by UV irradiation before immunization by a Th2 shift (data not shown). Our data support the idea that UV-B-induced immunosuppression is dependent on CD4⁺ Tregs, although the mechanism of immunosuppression induced by UV irradiation after immunization is not a Th2 shift.

Cytokines are important for UV-B-induced immunosuppression [11,12,27,42,53,64]. Especially, a role of IL-10 in high-dose UV-B irradiation was demonstrated [11,12,27,33,53,72]. High-dose UV-B is almost entirely absorbed within the epidermis [27], and UV-B irradiation causes damage to keratinocytes [27], which produce both IL-10 mRNA and protein *in vitro* [73], and thus are a likely source of IL-10 [27]. Apoptosis may also play an important role in UV-B-induced immunosuppression [1,74]. It is unlikely that UV-B-induced damage has a direct effect on T cells, because UV-B has a low penetration of skin [1]. It is considered that FasL expression on DNA-damaged LCs may stimulate Treg production, which may induce apoptosis in effector T cells [1]. FasL expression on DNA-damaged LCs may directly induce apoptosis of antigen-specific effector T cells [75]. Secretion of IL-4 and IL-10 from UV-B-induced apoptotic cells mediates immunosuppressive effects [1,76] and lymphocyte apoptosis is linked to IL-10 secretion [76]. In our model, possible sources of IL-10 may be apoptotic lymph nodes and/or damaged keratinocytes.

IL-10 is considered necessary for UV-B-induced immunosuppression [12,27,33,77–79]. Transferred IL-10-producing T cells inhibited the priming of alloantigen-specific CD4⁺T cells, and subsequent induction of alloantigen-specific CD8⁺T cells [33]. Transferred IL-10 CD4⁺T cells migrated to the site of allografts and directly inhibited the function of alloantigen-specific cytotoxic CD8⁺T cells [33]. The inhibitory capacity of UV-induced Tregs crucially depends on IL-10 [60]. Antigen-specific activation of Treg by APC induces the release of IL-10 [59,60] and inhibitory activity of UV-induced Treg is mediated by IL-10 [59,80]. From the viewpoint of the source of IL-10, UV-induced Treg itself [56,77], mast cells [81], and CD11b⁺ macrophages [82] were listed. Thus, IL-10 is crucial for both the induction and effector phases [11,33,73,76,78], but some researchers suggested that IL-10 is not required for Treg induction by UV irradiation [79]. IL-10 blocking data in the current study supports the importance of IL-10 for CD4⁺ Treg induction by immunization and UV-B irradiation.

Surprisingly, only CD4⁺T cells from immunized/UV-irradiated mice produced IL-10 when stimulated with the immunizing

alloantigen but not an irrelevant alloantigen. In this study, we used B6 mice as recipients, because B6 mice are Th1 prone [52]. This might explain why serum IL-10 was not detected. However, CD4⁺T cells from immunized and UV-irradiated B6 mice produced a large amount of IL-10 in an alloantigen-specific manner. High levels of IL-10 and low levels of IL-4 resemble a Tr1 cytokine pattern [83]. The presence of IL-10 gives rise to CD4⁺T-cell clones with a low proliferative capacity that in turn produce high levels of IL-10, low levels of IL-2, and no IL-4 [69,83]. These antigen-specific T cell clones suppress the proliferation of CD4⁺T cells in response to antigen [69,83]. Thus, IL-10 drives the generation of a CD4⁺T-cell subset, designated Tr1, which suppresses antigen-specific immune responses and actively down-regulates pathological immune responses *in vivo* [69,83]. Paradoxically, it may explain why T cells sorted by only CD4 well worked in our model. In our model, CD4⁺ Treg and a high level of IL-10 are important for alloantigen-specific immunosuppression.

Untreated control and immunized control showed similar survivals of allografts presenting the same alloantigen. Some questions arose. Although UV-irradiated control and immunized/UV-irradiated group showed the differences, does alloantigen immunization work well? Does stronger immunization provide more enhanced results? DC are specialized APCs that monitor the antigenic environment and activate naïve T cells [84]. The role of DC is to sense danger and tolerize the immune system to antigens encountered [84]. If naïve T cells encounter antigens on DC under certain conditions, they can differentiate into Tregs rather than effector T cells [84]. The induction of Tregs by DC in the presence of IL-10 has been documented [53,69,85,86], and bone marrow-derived DC can induce Tr1 differentiation [87,88]. Therefore, we initially expected that alloantigen immunization by bone marrow-derived DC would enhance alloantigen-specific immunosuppression. IL-10 is key for inducing and mediating tolerance [84], and UV-B-induced Tregs released a large amount of IL-10. We failed to enhance the immunosuppressive effect by using bone marrow-derived mature DC in our model, which might suggest the difficulty of memorization of alloantigen to UV-B-induced Tr1. A possible explanation for similar survivals between untreated and immunized controls was that transplanted allografts in this study had strong antigenicity.

Under the stimulation in MLC with anti-CD3 mAb, proliferation of responder CD4⁺T cells and IL-10 concentration in the supernatants were increased in all UV-irradiated groups. An interesting finding of this study is that high-dose UV-B irradiation alone seemed to result in pan-immunosuppression, although it was documented that UV-B-induced Tregs are highly selective for UV-B-induced cancer [2,37]. Moreover, we showed that the UV-B-induced pan-immunosuppressive effect was related to the time course. An explanation for this is that we used a

higher dose of UV-B (40 kJ/m²) than previous studies. In addition, the antigenicity of cardiac allografts might be weaker than for skin allografts, and thus some cardiac allografts survived even at 4 weeks after UV-B irradiation alone.

Interestingly, our results revealed that CD4⁺ Tregs induced by UV irradiation after immunization seemed to produce a subtle IL-10 in the stimulation with the irrelevant alloantigen. A possible explanation for this phenomenon was that UV-induced Tregs will show a unique behavior, so-called 'bystander suppression' [60,77,89]. The antigen specificity appears to be restricted to the activation of UV-induced Tregs and not to the suppressive activity itself, because once activated antigen-specifically, they release IL-10 and thereby suppress also other immune reactions. Further studies were required to explain this phenomenon, and we now are performing advanced studies in this DTH model by using the third-party alloantigen (H-2^{d/k}).

Furthermore, high-dose UV-B irradiation after alloantigen immunization, but not before immunization, is a useful tool for transferable Treg induction. We clearly demonstrated beneficial immunosuppression via alloantigen-specific Tr1-like CD4⁺T cells. UV-B irradiation to the recipient several days before transplantation is impractical, because predicting when a donor organ will be available is difficult. Alloantigen immunization before UV-B irradiation, suggested by the data here, may be clinically advantageous. The view of UV-induced immunology has changed over the past several years [3,4]. Carcinogenesis and immunosuppression due to UV were regarded as detrimental, but now it is thought that a fine-tuned balance is optimal [3,4]. In order to induce alloantigen-specific and transferable CD4⁺ Tr1 cells, UV is a very useful tool in the

DTH model. Alloantigen-specific immunosuppression is ideal for transplant recipients.

Clinically, there is great enthusiasm about the potential to develop strategies that can use Tregs for therapeutic intervention [77].

Conclusions

Induction of alloantigen-specific Tregs by alloantigen immunization and UV-B irradiation may have therapeutic potential. Although we are far from a full understanding of the mechanism involved, beneficial effects of alloantigen immunization and UV-B irradiation via alloantigen-specific Tr1-like CD4⁺T cells were clearly observed. This transferable Treg may be useful, especially in the field of transplant immunology. We hope that our new insights will help many researchers in the transplant immunology field.

Acknowledgments

We are very grateful to Masanori Niimi and Nozomu Shirasugi (Department of Surgery, Teikyo University Graduate School of Medicine, Tokyo, Japan) and Shintaro Shibutani (Department of Surgery, Keio University Graduate School of Medicine, Tokyo, Japan) for their technical support to establish ultra-microsurgery, especially for the murine transplantation model with cardiac allografts.

Conflict of interest

All authors had no financial conflict of interest.

References:

1. Aubin F, Mousson C: Ultraviolet light-induced regulatory (suppressor) T cells: an approach for promoting induction of operational allograft tolerance? *Transplantation*, 2004; 77: S29–31
2. Kripke ML: Immunological unresponsiveness induced by ultraviolet radiation. *Immunol Rev*, 1984; 80: 87–102
3. Ullrich SE, Byrne SN: The immunologic revolution: photoimmunology. *J Invest Dermatol*, 2012; 132: 896–905
4. Schwarz T, Beissert S: Milestones in photoimmunology. *J Invest Dermatol*, 2013; 133: E7–10
5. Myakishev-Rempel M, Kuper J, Mintz B et al: Investigation of the peak action wavelength of light-activated gene transduction. *Gene Ther*, 2011; 18: 1043–51
6. Spellman CW, Woodward JG, Daynes RA: Modification of immunological potential by ultraviolet radiation. I. Immune status of short-term UV-irradiated mice. *Transplantation*, 1977; 24: 112–19
7. Ullrich SE, Magee M: Specific suppression of allograft rejection after treatment of recipient mice with ultraviolet radiation and allogeneic spleen cells. *Transplantation*, 1988; 46: 115–19
8. Lau H, Reemtsma K, Hardy MA: Pancreatic islet allograft prolongation by donor-specific blood transfusions treated with ultraviolet irradiation. *Science*, 1983; 221: 754–56
9. Lau H, Reemtsma K, Hardy MA: Prolongation of rat islet allograft survival by direct ultraviolet irradiation of the graft. *Science*, 1984; 223: 607–9
10. Magee MJ, Kripke ML, Ullrich SE: Suppression of the elicitation of the immune response to alloantigen by ultraviolet radiation. *Transplantation*, 1989; 47: 1008–13
11. Nghiem DX, Walterscheid JP, Kazimi N, Ullrich SE: Ultraviolet radiation-induced immunosuppression of delayed-type hypersensitivity in mice. *Methods*, 2002; 28: 25–33
12. Dupont E, Craciun L: UV-induced immunosuppressive and anti-inflammatory actions: mechanisms and clinical applications. *Immunotherapy*, 2009; 1: 205–10
13. Azzi JR, Sayegh MH, Mallat SG: Calcineurin inhibitors: 40 years later, can't live without. *J Immunol*, 2013; 191: 5785–91
14. Schwartz RH: Natural regulatory T cells and self-tolerance. *Nat Immunol*, 2005; 6: 327–30
15. Bluestone JA: Regulatory T-cell therapy: is it ready for the clinic? *Nat Rev Immunol*, 2005; 5: 343–49
16. Walsh PT, Taylor DK, Turka LA: Tregs and transplantation tolerance. *J Clin Invest*, 2004; 114: 1398–403
17. Plain KM, Verma ND, Tran GT et al: Cytokines affecting CD4⁺ T regulatory cells in transplant tolerance. Interleukin-4 does not maintain alloantigen specific CD4⁺CD25⁺ Treg. *Transpl Immunol*, 2013; 29: 51–59
18. Starzl TE, Murase N, Abu-Elmagd K et al: Tolerogenic immunosuppression for organ transplantation. *Lancet*, 2003; 361: 1502–10

19. Nishimura E, Sakihama T, Setoguchi R et al: Induction of antigen-specific immunologic tolerance by *in vivo* and *in vitro* antigen-specific expansion of naturally arising Foxp3⁺CD25⁺CD4⁺ regulatory T cells. *Int Immunol*, 2004; 16: 1189–201
20. Joffre O, Santolaria T, Calise D et al: Prevention of acute and chronic allograft rejection with CD4⁺CD25⁺Foxp3⁺ regulatory T lymphocytes. *Nat Med*, 2008; 14: 88–92
21. Kato T, Wang L: UV-Induced Immune Suppression that Promotes Skin Cancer Development and Progression. In: La Porta C (ed.), *Skin Cancers – Risk Factors, Prevention and Therapy*. Rijeka: InTech; 2011
22. Toda M, Wang L, Ogura S et al: UV irradiation of immunized mice induces type 1 regulatory T cells that suppress tumor antigen specific cytotoxic T lymphocyte responses. *Int J Cancer*, 2011; 129: 1126–36
23. Wang L, Saito K, Toda M et al: UV irradiation after immunization induces type 1 regulatory T cells that suppress Th2-type immune responses via secretion of IL-10. *Immunobiology*, 2010; 215: 124–32
24. Wang L, Toda M, Saito K et al: Post-immune UV irradiation induces Tr1-like regulatory T cells that suppress humoral immune responses. *Int Immunol*, 2008; 20: 57–70
25. Hori T, Kuribayashi K, Uemoto S et al: Alloantigen-specific prolongation of allograft survival in recipient mice treated by alloantigen immunization following ultraviolet-B irradiation. *Transpl Immunol*, 2008; 19: 45–54
26. Ullrich SE: Suppression of the immune response to allogeneic histocompatibility antigens by a single exposure to ultraviolet radiation. *Transplantation*, 1986; 42: 287–91
27. Beissert S, Hosoi J, Kuhn R et al: Impaired immunosuppressive response to ultraviolet radiation in interleukin-10-deficient mice. *J Invest Dermatol*, 1996; 107: 553–57
28. Mottram PL, Mirisklavos A, Clunie GJ, Noonan FP: A single dose of UV radiation suppresses delayed type hypersensitivity responses to alloantigens and prolongs heart allograft survival in mice. *Immunol Cell Biol*, 1988; 66: 377–85
29. Ullrich SE: Two-way traffic on the bridge from innate to adaptive immunity. *J Invest Dermatol*, 2010; 130: 1773–75
30. Greene MI, Sy MS, Kripke M, Benacerraf B: Impairment of antigen-presenting cell function by ultraviolet radiation. *Proc Natl Acad Sci USA*, 1979; 76: 6591–95
31. Noonan FP, Kripke ML, Pedersen GM, Greene MI: Suppression of contact hypersensitivity in mice by ultraviolet irradiation is associated with defective antigen presentation. *Immunology*, 1981; 43: 527–33
32. Granstein RD: Epidermal I-J-bearing cells are responsible for transferable suppressor cell generation after immunization of mice with ultraviolet radiation-treated epidermal cells. *J Invest Dermatol*, 1985; 84: 206–9
33. Miyamoto T, Kaneko T, Yamashita M et al: Prolonged skin allograft survival by IL-10 gene-introduced CD4 T cell administration. *Int Immunol*, 2005; 17: 759–68
34. Niimi M: The technique for heterotopic cardiac transplantation in mice: experience of 3000 operations by one surgeon. *J Heart Lung Transplant*, 2001; 20: 1123–28
35. Shibutani S, Inoue F, Aramaki O et al: Effects of immunosuppressants on induction of regulatory cells after intratracheal delivery of alloantigen. *Transplantation*, 2005; 79: 904–13
36. Kato T, Nariuchi H: Polarization of naive CD4⁺ T cells toward the Th1 subset by CTLA-4 costimulation. *J Immunol*, 2000; 164: 3554–62
37. Kripke ML: Antigenicity of murine skin tumors induced by ultraviolet light. *J Natl Cancer Inst*, 1974; 53: 1333–36
38. Daynes RA, Spellman CW: Evidence for the generation of suppressor cells by ultraviolet radiation. *Cell Immunol*, 1977; 31: 182–87
39. Fisher MS, Kripke ML: Further studies on the tumor-specific suppressor cells induced by ultraviolet radiation. *J Immunol*, 1978; 121: 1139–44
40. Polla L, Margolis R, Goulston C et al: Enhancement of the elicitation phase of the murine contact hypersensitivity response by prior exposure to local ultraviolet radiation. *J Invest Dermatol*, 1986; 86: 13–17
41. Molendijk A, van Gorp RJ, Donselaar IG, Benner R: Suppression of delayed-type hypersensitivity to histocompatibility antigens by ultraviolet radiation. *Immunology*, 1987; 62: 299–305
42. Boonstra A, van Oudenaren A, Barendregt B et al: UVB irradiation modulates systemic immune responses by affecting cytokine production of antigen-presenting cells. *Int Immunol*, 2000; 12: 1531–38
43. Byrne SN, Limon-Flores AY, Ullrich SE: Mast cell migration from the skin to the draining lymph nodes upon ultraviolet irradiation represents a key step in the induction of immune suppression. *J Immunol*, 2008; 180: 4648–55
44. Stamatas GN, Morello AP, Mays DA: Early inflammatory processes in the skin. *Curr Mol Med*, 2013; 13(8): 1250–69
45. Schwarz A, Maeda A, Kernebeck K et al: Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair. *J Exp Med*, 2005; 201: 173–79
46. Fukunaga A, Khaskhely NM, Ma Y et al: Langerhans cells serve as immunoregulatory cells by activating NKT cells. *J Immunol*, 2010; 185: 4633–40
47. Toews GB, Bergstresser PR, Streilein JW: Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J Immunol*, 1980; 124: 445–53
48. Romani N, Brunner PM, Stingl G: Changing views of the role of Langerhans cells. *J Invest Dermatol*, 2012; 132: 872–81
49. Streilein JW, Toews GT, Gilliam JN, Bergstresser PR: Tolerance or hypersensitivity to 2,4-dinitro-1-fluorobenzene: the role of Langerhans cell density within epidermis. *J Invest Dermatol*, 1980; 74: 319–22
50. Chacon-Salinas R, Chen L, Chavez-Blanco AD et al: An essential role for platelet-activating factor in activating mast cell migration following ultraviolet irradiation. *J Leukoc Biol*, 2014; 95: 139–48
51. Rocha PN, Plumb TJ, Crowley SD, Coffman TM: Effector mechanisms in transplant rejection. *Immunol Rev*, 2003; 196: 51–64
52. Csencsits K, Wood SC, Lu G et al: Graft rejection mediated by CD4⁺ T cells via indirect recognition of alloantigen is associated with a dominant Th2 response. *Eur J Immunol*, 2005; 35: 843–51
53. Graca L, Thompson S, Lin CY et al: Both CD4⁺CD25⁺ and CD4⁺CD25⁻ regulatory cells mediate dominant transplantation tolerance. *J Immunol*, 2002; 168: 5558–65
54. Elmetts CA, Bergstresser PR, Tigelaar RE et al: Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J Exp Med*, 1983; 158: 781–94
55. Fisher MS, Kripke ML: Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice. *Science*, 1982; 216: 1133–34
56. Schwarz T: 25 years of UV-induced immunosuppression mediated by T cells from disregarded T suppressor cells to highly respected regulatory T cells. *Photochem Photobiol*, 2008; 84: 10–18
57. Loser K, Beissert S: Regulatory T cells: banned cells for decades. *J Invest Dermatol*, 2012; 132: 864–71
58. Beissert S, Schwarz A, Schwarz T: Regulatory T cells. *J Invest Dermatol*, 2006; 126: 15–24
59. Schwarz A, Beissert S, Grosse-Heitmeyer K et al: Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. *J Immunol*, 2000; 165: 1824–31
60. Schwarz A, Maeda A, Wild MK et al: Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. *J Immunol*, 2004; 172: 1036–43
61. Krasteva M, Aubin F, Laventurier S et al: MHC class II-KO mice are resistant to the immunosuppressive effects of UV light. *Eur J Dermatol*, 2002; 12: 10–19
62. Nghiem DX, Kazimi N, Mitchell DL et al: Mechanisms underlying the suppression of established immune responses by ultraviolet radiation. *J Invest Dermatol*, 2002; 119: 600–8
63. Bromley SK, Yan S, Tomura M et al: Recirculating memory T cells are a unique subset of CD4⁺ T cells with a distinct phenotype and migratory pattern. *J Immunol*, 2013; 190: 970–76
64. Rivas JM, Ullrich SE: The role of IL-4, IL-10, and TNF-alpha in the immune suppression induced by ultraviolet radiation. *J Leukoc Biol*, 1994; 56: 769–75
65. Ullrich SE: Does exposure to UV radiation induce a shift to a Th-2-like immune reaction? *Photochem Photobiol*, 1996; 64: 254–58
66. Gorgun G, Miller KB, Foss FM: Immunologic mechanisms of extracorporeal photochemotherapy in chronic graft-versus-host disease. *Blood*, 2002; 100: 941–47
67. Blom L, Poulsen LK: *In vitro* Th1 and Th2 cell polarization is severely influenced by the initial ratio of naive and memory CD4⁺ T cells. *J Immunol Methods*, 2013; 397: 55–60
68. Mazzarella G, Bianco A, Catena E et al: Th1/Th2 lymphocyte polarization in asthma. *Allergy*, 2000; 55: 6–9

69. Groux H, Bigler M, de Vries JE, Roncarolo MG: Interleukin-10 induces a long-term antigen-specific anergic state in human CD4⁺ T cells. *J Exp Med*, 1996; 184: 19–29
70. Letterio JJ, Roberts AB: Regulation of immune responses by TGF-beta. *Annu Rev Immunol*, 1998; 16: 137–61
71. Cottrez F, Groux H: Regulation of TGF-beta response during T cell activation is modulated by IL-10. *J Immunol*, 2001; 167: 773–78
72. Schwarz A, Grabbe S, Riemann H et al: *In vivo* effects of interleukin-10 on contact hypersensitivity and delayed-type hypersensitivity reactions. *J Invest Dermatol*, 1994; 103: 211–16
73. Rivas JM, Ullrich SE: Systemic suppression of delayed-type hypersensitivity by supernatants from UV-irradiated keratinocytes. An essential role for keratinocyte-derived IL-10. *J Immunol*, 1992; 149: 3865–71
74. Salucci S, Burattini S, Battistelli M et al: Ultraviolet B (UVB) irradiation-induced apoptosis in various cell lineages *in vitro*. *Int J Mol Sci*, 2012; 14(1): 532–46
75. Hill LL, Shreedhar VK, Kripke ML, Owen-Schaub LB: A critical role for Fas ligand in the active suppression of systemic immune responses by ultraviolet radiation. *J Exp Med*, 1999; 189: 1285–94
76. Tomimori Y, Ikawa Y, Oyaizu N: Ultraviolet-irradiated apoptotic lymphocytes produce interleukin-10 by themselves. *Immunol Lett*, 2000; 71: 49–54
77. Maeda A, Beissert S, Schwarz T, Schwarz A: Phenotypic and functional characterization of ultraviolet radiation-induced regulatory T cells. *J Immunol*, 2008; 180: 3065–71
78. Loser K, Apel J, Voskort M et al: IL-10 controls ultraviolet-induced carcinogenesis in mice. *J Immunol*, 2007; 179: 365–71
79. Ghoreishi M, Dutz JP: Tolerance induction by transcutaneous immunization through ultraviolet-irradiated skin is transferable through CD4⁺CD25⁺ T regulatory cells and is dependent on host-derived IL-10. *J Immunol*, 2006; 176: 2635–44
80. Maeda A, Schwarz A, Bullinger A et al: Experimental extracorporeal photopheresis inhibits the sensitization and effector phases of contact hypersensitivity via two mechanisms: generation of IL-10 and induction of regulatory T cells. *J Immunol*, 2008; 181: 5956–62
81. Chacon-Salinas R, Limon-Flores AY, Chavez-Blanco AD et al: Mast cell-derived IL-10 suppresses germinal center formation by affecting T follicular helper cell function. *J Immunol*, 2011; 186: 25–31
82. Kang K, Hammerberg C, Meunier L, Cooper KD: CD11b⁺ macrophages that infiltrate human epidermis after *in vivo* ultraviolet exposure potentially produce IL-10 and represent the major secretory source of epidermal IL-10 protein. *J Immunol*, 1994; 153: 5256–64
83. Groux H, O'Garra A, Bigler M et al: A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*, 1997; 389: 737–42
84. Levings MK, Gregori S, Tresoldi E et al: Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25⁺CD4⁺ Tr cells. *Blood*, 2005; 105: 1162–69
85. Hara M, Kingsley CI, Niimi M et al: IL-10 is required for regulatory T cells to mediate tolerance to alloantigens *in vivo*. *J Immunol*, 2001; 166: 3789–96
86. Kingsley CI, Karim M, Bushell AR, Wood KJ: CD25⁺CD4⁺ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J Immunol*, 2002; 168: 1080–86
87. Wakkach A, Fournier N, Brun V et al: Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation *in vivo*. *Immunity*, 2003; 18: 605–17
88. Brinster C, Shevach EM: Bone marrow-derived dendritic cells reverse the anergic state of CD4⁺CD25⁺ T cells without reversing their suppressive function. *J Immunol*, 2005; 175: 7332–40
89. Groux H, Powrie F: Regulatory T cells and inflammatory bowel disease. *Immunol Today*, 1999; 20: 442–45